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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c)

Atty Ref No. 038586-330; Client Ref. No. 2003-471-1

INVENTOR(s)		
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TITLE OF THE INVENTION (280 characters max)

BONE MORPHOGENIC PROTEIN BINDING PROTEIN

☑ *Additional inventors are being named on the __1__ separately numbered sheets attached hereto*

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ENCLOSED APPLICATION PARTS (*CHECK ALL THAT APPLY*)

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☑ Applicant claims small entity status. See 37 C.F.R. 1.27	PROVISIONAL FILING FEE AMOUNT	$80.00
☐ A check or money order is enclosed to cover the filing fees		
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The Invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ 4 No

☐ 5 Yes, the name of the U.S Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE _____ Date January 28, 2004

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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U.S. Provisional Application

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PROVISIONAL PATENT APPLICATION
UNDER 37 C.F.R. 1.53(C)

For

BONE MORPHOGENIC PROTEIN BINDING PROTEIN

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UC Matter 2003-471-1

BONE MORPHOGENIC PROTEIN (BMP) BINDING PROTEIN

BACKGROUND OF THE INVENTION

[0001] Field of Invention

[0002] The invention generally relates to calcifying and osteogenic factors, and more particularly to the peptide "BMP Binding Protein" (BBP), to compositions including BBP, articles of manufacture including BBP and methods of using the compositions and articles and induce calcification and osteogenesis.

[0003] Growth factors are substances, such as peptides, which affect the growth and differentiation of defined populations of animal cells *in vivo* or *in vitro*.

[0004] Normal bone formation occurs during development and remodeling occurs in adult life in order to preserve the integrity of the skeleton. Bone formation and remodeling involves bone resorption by osteoclasts and bone formation by osteoblasts, which are regulated by growth factors. Thus, any interference between the balance in cell differentiation bone formation and bone resorption can affect bone homeostasis, bone formation and repair.

[0005] The osteoblasts are derived from a pool of marrow stromal cells (also known as mesenchymal stem cells; MSC). These cells are present in a variety of tissues and are prevalent in bone marrow stroma. MSC are pluripotent and can differentiate into osteogenic cells including osteoblasts, chondrocytes, fibroblasts, myocytes, and adipocytes.

[0006] One family of growth factors have been described as bone morphogenic protein/non-collagenous protein (BMP/NCP or BMPs), which are in the TGF-β family of proteins.

[0007] Researchers previously described a partially purified extract of demineralized bone matrix which induced the formation of ectopic bone. This substance, BMP/NCP was never purified to homogeneity, but other investigators have used similar starting materials to clone a number of recombinant "BMPs." Oddly, several of these "BMPs" have little or no osteogenic activity.

[0008] "BMPs" and other osteogenic factors have been studied for use in clinical applications. However, the cost of using minimally effective dosages of BMP has been a limiting factor in clinical use.

[0009] Safe, effective and affordable compositions and methods are desired to treat bone disorders (such as osteoporosis), bone injury (such as fracture healing flat (e.g., membranous) and long (e.g., endochondral) bones, non-union fractures and reconstructive surgery). The invention may also be used in treating periodontitis, periodontal regeneration, alveolar ridge augmentation for tooth implant reconstruction, treatment of non-union fractures, sites of knee/hip/joint repair or replacement surgery.

[0010] Further, the use of compositions and methods in combination with other methods for enhancing calcification and osteogenesis is desirable.

[0011] Specifically, peptides are useful agents, particularly those that are secreted due to their ability to be used in physiologically active, soluble forms. These factors, their receptors, DNA and RNA coding sequences, and fragments thereof are useful in a number of therapeutic, clinical, research, diagnostic and drug design applications.

BRIEF SUMMARY OF INVENTION

[0012] The invention may include:

1. A substantially pure protein comprising the amino acid sequence of SEQ ID No: 1a.

2. A substantially pure protein comprising the amino acid sequence of SEQ ID No: 1a or a fragment thereof, wherein said fragment has calcification activity in mammalian cells.

3. A composition comprising a substantially pure protein and a physiologically acceptable carrier with which the protein is admixed, wherein said protein comprises either the amino acid sequence of SEQ ID No: 1a or a fragment thereof, wherein said fragment has calcifying activity mammalian cells.

4. A composition comprising a physiologically acceptable carrier, a protein comprising the either the amino acid sequence of SEQ ID No: 1a or a fragment

thereof, and BMP-2 or demineralized bone matrix wherein said fragment has osteogenic activity mammalian cells.

5. An isolated DNA encoding a functional protein, and having the amino acid sequence of SEQ ID No: 1a.

6. A construct comprising DNA encoding a functional protein, and having the amino acid sequence of SEQ ID No: 1a operatively linked to an expression vector.

7. A transformant obtained by introducing the construct of claim 6 into a host.

8. The DNA as in claim 5 wherein the protein encoded induces osteogenesis in vertebrates.

9. An antibody to BPP, or a fragment thereof, with selective binding to BPP.

10. An antibody to BPP, or a fragment thereof, wherein the antibody inhibits calcification or osteogenesis.

11. A method for producing a purified BBP comprising the steps of:

(a) culturing a host cell transformed with a DNA molecule DNA encoding a functional protein, and having the amino acid sequence of SEQ ID NO: 1a; and

(b) recovering and purifying the BPP from the culture medium.

12. A medicament for use in enhancing calcification comprising a therapeutically effective dosage of BBP.

13. A medicament for use in osteogenesis, comprising a therapeutically effective dosage of BBP and one of BMP-2 or demineralized bone matrix.

14. The medicament of claim 12 or 13, further comprising at least one agent selected from the group comprising parathyroid hormone, sodium fluoride, insulin-like

growth factor I, insulin-like growth factor II or transforming growth factor beta, bisphosphonates, selective estrogen receptor modulators, calcitonin, vitamin D or calcium.

15. A method of inducing calcification including treating a mammalian cell with BBP.

16. A method of inducing osteogenesis including treating a mammalian cell with BBP and one of BMP-2 or demineralized bone matrix.

17. The method of claim 11, further comprising treating the mammalian cells with at least one agent selected from the group comprising parathyroid hormone, sodium fluoride, insulin-like growth factor I, insulin-like growth factor II or transforming growth factor beta, bisphosphonates, selective estrogen receptor modulators, calcitonin, or vitamin D and calcium, at a therapeutically effective dose.

18. A method of stimulating mammalian cells to express a level of a biological marker of calcification which is greater than the level of a biological marker in untreated cells, comprising exposing a mammalian cells to BBP.

19. A method of stimulating mammalian cells to express a level of a biological marker of osteogenesis which is greater than the level of a biological marker in untreated cells, comprising exposing a mammalian cells to BBP and one of BMP-2 or demineralized bone matrix.

20. The method of claim 18 wherein the biological marker is an increase in at least one of alkaline phosphatase activity, calcium incorporation, mineralization or expression of osteocalcin mRNA.

21. A method of treating a patient to increase calcification of endochondral bone, comprising administering BBP at a therapeutically effective dose in an effective dosage form at a selected interval to enhance calcification.

22. A method of treating a patient to increase osteogenesis of endochondral bone, comprising administering BBP at a therapeutically effective dose in an effective dosage form at a selected interval and one of BMP-2 or demineralized bone matrix to enhance osteogenesis.

23. A method of treating a patient to induce bone formation comprising: administering osteogenic cells to the patient at a location proximate to the desired location of osteogenesis; and administering BBP at a location proximate to the desired location of osteogenesis.

24. The method of claim 23, further comprising treating the patient with at least one agent selected from the group comprising BMP or demineralized bone matrix.

25. An article of manufacture comprising a protein immobilized on a solid support, wherein said protein comprises either the amino acid sequence of sequence of SEQ ID No: 1a or a fragment thereof, wherein said fragment has calcifying activity.

26. An article of manufacture of claim 25, further comprising a protein immobilized on a solid support, wherein said protein comprises either the amino acid sequence of sequence of BMP-2 or a fragment thereof, wherein said fragment has osteogenesis activity.

27. An implant for use *in vivo* comprising, a substrate having a surface, wherein at least the surface of the implant includes BBP in an amount sufficient to enhance calcification.

28. The implant of claim 27, wherein the substrate is formed into the shape of a pin, screw, plate, or prosthetic joint.

29. The implant of claim 27, wherein at least the surface of the implant includes osteogenic mammalian cells.

[0013] The bovine derived SEQ ID No: 1a has been designated "BBP," and this peptide is capable of inducing calcification in mammals. SEQ ID No: 1b corresponds to the nucleic acid sequence encoding BBP. Since peptide of the invention induces osteogenesis in vertebrates, it should be able to be prepared in physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

[0014] BBP is a 19 amino acid, 2.1kD peptide, derived from a 18.5 kD fragment of a known 24 kDa secreted phosphoprotein, SPP-24. The amino acid sequence which contains BBP, is illustrated by SEQ ID No: 2. No function for SPP-24 has ever been published. BBP contains the cystatin-like domain of SPP-24. A 19 amino acid region of the peptide is similar to the TGF-β/BMP-binding region of fetuin, a member of the cystatin family of protease inhibitors. The peptide avidly bounds rhBMP-2 (recombinant human BMP-2) with a K_D of x 10^{-5} M. BBP may also bind related molecules (from the transforming growth factor-beta family of proteins) and affect their function as well. BBP is expressed at least in the liver and bone (including demineralized cortical bone and periosteum).

[0015] BBP alone enhances calcification and increases the osteogenic activity of recombinant BMP, demineralized bone matrix containing BMP and additional osteogenic agents. Surprisingly, BBP as used with BMP *in vivo* causes osteogenesis to occur faster and to a greater extent and with smaller amounts of rhBMP-2.

[0016] For example, when implanted alone in mouse muscle, the BBP induces dystrophic calcification. When BBP is implanted with rhBMP-2, BBP enhanced chondrogenesis and osteogenic activity of the recombinant molecule.

[0017] BBP or fragments thereof (which also may be synthesized by *in vitro* methods) may be fused (by recombinant expression or *in vitro* covalent methods) to an immunogenic polypeptide and this, in turn, may be used to immunize an animal in order to raise antibodies against the novel proteins. Antibodies are recoverable from the serum of immunized animals. Alternatively, monoclonal antibodies may be prepared from cells from the immunized animal in conventional fashion. Immobilized antibodies are useful particularly in the diagnosis (*in vitro* or *in vivo*) or purification of BBP.

[0018] Two examples of specific peptide sequences against which rabbit polyclonal antibodies were generated include:

[0019] 1. An antibody against the peptide sequence "IQETTCRRESEADPATCDFQRGYHVPVAVCRSTVRMSAEQV" that reacts with both bovine and human secreted phosphoprotein 24. This antibody was generated in rabbits immunized with the synthetic peptide indicated above.

[0020] 2. An antibody directed against the sequence "CGEPLYEPSREMRRN" that was also produced in rabbits immunized with a synthetic peptide corresponding to the indicated sequence. This antibody reacts with bovine secreted phosphoprotein 24. The N-terminal cysteine is not a part of the native secreted phosphoprotein 24 sequence; but is preferably included to allow the peptide to be conjugated to chromatographic resins for affinity chromatography.

[0021] Antibodies against those sequences, corresponding sequences in the mouse, human, and rat genome, or any derivatives of the immunogenic sequences are also useful in this invention. These antibodies are useful in at least to the extent that they recognize the native BBP molecule with high specificity. Such antibodies may also be useful in inhibiting protein specific interactions of BBP with other molecules where the antibody binds to a location on the peptide which interacts with other molecules. The inhibition of BBP activity in situations where the rate or degree of chondogenesis or osteogenesis may be modified.

[0022] BBP also may be derivatized *in vitro* in order to prepare immobilized and labeled proteins, particularly for purposes of diagnosis of insufficiencies thereof, or for affinity purification of antibodies thereto.

[0023] Substitutional, deletional, or insertional mutants of BBP may be prepared by *in vitro* or recombinant methods and screened for immuno-crossreactivity with BBP and for BBP antagonist or agonist activity.

[0024] The invention may further include any portion of the BBP peptide which is found to be active in affecting calcification or osteogenesis. The invention may also include mutations of the BBP sequence which are found to be active in affecting calcification or osteogenesis. For example, conservative and semi-conservative

substitution may occur naturally or between species which may enhance or diminish the effect of the activity of the peptide.

[0025] The invention may include a method of systemic delivery or localized treatment with agents for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair. The invention may include a method of systemic delivery or localized treatment with osteogenic cells for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair.

[0026] In one application of the invention, the method may be applied to induce the local repair of bone or to treat bone related disorders, such as osteoporosis.

[0027] The invention may also include implants having coatings of substances or seeded with differentiated cells for inducing bone formation or enhancing bone repair. The invention may also include the application of substances or differentiated cells at a site where bone formation or bone repair is desired. For example, implants may include, but are not limited to pins, screws and plates that are used to immobilize a fracture, enhance bone formation or stabilize a prosthetic implant by stimulating bone formation or bone repair.

[0028] This invention is advantageous at least in that BBP enhances calcification and the activity of other osteogenic materials, such as BMP. Therefore, lower doses of osteogenic materials may be used for clinical applications. This is significant at least in that clinical treatments may be more affordable. Further this invention is advantageous at least in that BBP enhances osteogenesis to occur faster to a greater extent, which may improve the clinical rate and effectiveness of treatment with BMP.

[0029] These, as well as other objects, features and benefits will now become clear from a review of the following detailed description of illustrative embodiments and the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

[0030] FIG. 1A (1) and (2) include the bovine amino acid and nucleic acid sequences, respectively of BBP; FIG. 1B is a partial amino acid sequence of the bovine BMP binding protein ("BBP") showing the cystatin homology region, the BMP-2 similarity region, and the TGF-β receptor II homology domain. Underlined amino acids have

been confirmed to be present by mass spectroscopy. (GenBank Accession Number U08018; Hu, B et al., J. Biol. Chem. 270:431-436, 1995).

[0031] FIG. 2 is a sequence alignment of human BMP-2 and the BMP-2 similarity region in SPP-24. (i, identical; c, conservative substitution; sc, semi-conservative substitution)

[0032] FIG. 3 is a sequence alignment of bovine fetuin and human TGF-β receptor II (above) and of human TGF-β receptor II and the TGF-β receptor II homology domain of bovine SPP-24 (corresponding to BBP). (i, identical; c, conservative substitution; sc, semi-conservative substitution).

[0033] FIG. 4 is a radiogram of mouse hind quarters 21 days after implantation of 500 μg of BBP in atelocollagen (above) or atelocollagen alone (below). Note the calcification.

[0034] FIG. 5 is a histological section of mouse muscle 21 days after implantation of 500 μg of BBP in atelocollagen. Note the dystrophic calcification primarily associated with intramuscular adipose tissue. H & E stain. Original magnification 100 X.

[0035] FIG. 6 are radiograms of mouse hind quarters 21 days after implantation of 5 μg of rhBMP-2 (left) or 5 μg of rhBMP-2 plus 500 mg of BBP (right). Note the increased opacity associated with the samples containing both rhBMP-2 and BBP.

[0036] FIG. 7 are radiograms of mouse hind quarters 9 (above) and 12 (below) days after implantation of 5 μg of rhBMP-2 (left) or 5 μg of rhBMP-2 plus 500 mg of BBP (right). Note the appearance of calcification in the sample from the day 9 sample containing both rhBMP-2 and BBP but not the sample containing BMP-2 alone.

[0037] FIG. 8 are histological sections of mouse hind quarters 9 days after implantation of 5 μg of rhBMP-2 alone (A) or 5 μg of rhBMP-2 plus 500 μg of BBP (B). Note the abundant cartilage in the BMP + BBP specimen whereas the BMP alone specimen shows the earlier stages of inflammation and mesodermal cell proliferation.

[0038] FIG. 9 is a surface plasmon resonance sensogram for the interaction of rhBMP-2 (affixed to the chip) and cyclized BBP at concentrations ranging from 1×10^{-5} M 1×10^{-4} M.

[0039] FIG. 10 are representative frontal sections of the distal femoral metaphysis from 3 month old female wild type (A) and transgenic (B) mice. The transgenic animals show reduced femoral cancellous bone mass accompanied by reduced trabecular connection and increased trabecular separation. Five micrometer-thick undecalcified sections. Masson-Goldner trichrome stain. bar = 100 μm.

[0040] FIG. 11 are histologic comparisons of trabecular thickness in wild type (A) and transgenic (B) female mice. Note the reduced in cancellous bone volume in females transgenic animals that is associated with a reduction in individual trabeculae thickness. Five micrometer-thick undecalcified sections. Masson-Goldner trichrome stain. bar = 50 μm.

[0041] FIG. 12 is a flow chart of one method of the invention.

[0042] FIGS. 13 A & B are schematic depictions of two embodiments of the present invention.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0043] A number of applications for BBP are suggested from its pharmacological (biological activity) properties.

[0044] For example, the BPP amino acids and nucleic acids can be useful as a diagnostic tool (such as through use of antibodies in assays, for expressing protein in cell lines or use of oligonucleotides as primers in a PCR test to amplify those with sequence similarities to the oligonucleotide primer, and to determine how much of the novel protein is present).

[0045] BBP might act upon its target cells via its own receptor or by binding other molecules, such as BMP. BBP, therefore, provides the key to isolate this receptor. Thus, when one views BBP as ligand in complexes, then complexes in accordance with the invention include antibody bound to BBP, antibody bound to peptides derived from BBP, BBP bound to its receptor, or peptides derived from BBP bound to its receptor or other factors. Mutant forms of BBP, which are either more potent agonists or antagonists, are believed to be clinically useful. Such complexes of BBP and its binding protein partners will find uses in a number of applications.

[0046] Practice of this invention includes use of an oligonucleotide construct comprising a sequence coding for BBP and for a promoter sequence operatively linked in a mammalian or a viral expression vector. Expression and cloning vectors contain a nucleotide sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomes, and includes origins of replication or autonomously replicating sequences. Such cloning vectors are well known to those of skill in the art.

[0047] Expression and cloning vectors should contain a selection gene, also termed a selectable marker. Typically, this is a gene that encodes a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies.

[0048] Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR) or thymidine kinase. Such markers enable the identification of cells which were competent to take up the BBP nucleic acid. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of BBP can therefore be synthesized from the amplified DNA.

[0049] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium which contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell deficient in DHFR activity, prepared and propagated as described by Urlaub and

Chasin, Proc. Nat. Acac. Sci., 77, 4216 (1980). The transformed cells then are exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding BBP. Alternative methods of expression and selection are known by those skilled in the art.

[0050] Expression vectors, unlike cloning vectors, may contain a promoter which is recognized by the host organism and is operably linked to the BBP nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters can be operably linked to BBP encoding DNA by removing them from their gene of origin by restriction enzyme digestion, followed by insertion 5' to the start codon for BBP or BMP.

[0051] Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exit then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

[0052] Transcription of the protein-encoding DNA in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma,

cytomegalovirus, adenovirus, retroviruses, hepatitis-B virus, and most preferably Simian Virus 40 (SV40), or from heterologous mammalian promoters, e.g. the actin promoter. Of course, promoters from the host cell or related bovine also are useful herein.

[0053] Polyclonal antibodies to BPP generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of BBP and an adjuvant. It may be useful to conjugate these proteins or a fragment containing the target amino acid sequence to a protein which is immunogenic in the bovine to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl.sub.2, or R.sup.1 N.dbd.C.dbd.NR.

[0054] Animals can be immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 .mu.g of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally in multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Fruend's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for anti-BBP titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same BBP polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

[0055] Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with mycelia cells or by BE virus transformation and screening for clones expressing the desired antibody.

[0056] Antibodies are useful in diagnostic assays for BBP or their antibodies and to identify family members. In one embodiment of a receptor binding assay, an antibody

composition which binds to all of a selected plurality of members of the BBP family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition in order to adsorb all BBP family members, and then the immobilized family members are contacted with a plurality of antibodies specific for each member, each of the antibodies being individually identifiable as specific for a predetermined family member, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member can be determined.

[0057] The antibodies also are useful for the affinity purification of the BBP from recombinant cell culture or natural sources. Antibodies that do not detectably cross-react with other growth factors can be used to purify the proteins free from these other family members.

[0058] Further, the present invention is related to BBP and methods for maintaining bone homeostasis, enhancing calcification or osteogenesis to promote bone formation and/or enhancing bone repair.

[0059] More specifically, the invention may include the systemic and/or local application of agents for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair. Clinical indices of a method or compounds ability to maintain bone homeostasis is evidenced by improvements in bone density at different sites through out the body as assessed, at least by DEXA scanning. Enhanced bone formation in a healing fracture is routinely assessed by regular X-ray of the fracture site at selected time intervals. More advanced techniques for determining the above indices, such as quantitative CT scanning may be used.

[0060] More specifically, the invention may include the use of agents which stimulate osteogenesis. The invention may include the use of agents which influence the differentiation of osteoblasts or osteoclasts.

[0061] The invention may include the use of agents which inhibit osteoclastic bone resorption. Agents which may be useful in this invention to effect osteoclastic bone resorption include, but are not limited to, bisphosphonates, the selective estrogen receptor modulators, calcitonin, and vitamin D/calcium supplementation. The

invention may also include the use of agents which induce osteoblastic bone formation. Agents which may be useful in this invention include, but are not limited to PTH, sodium fluoride and growth factors, such as insulin-like growth factors I and II and transforming growth factor beta.

[0062] The *in vivo* models used to show the calcification effects of BBP alone or osteogenic effects in combination with BMP have been used previously in demonstrating similar behaviors of other compounds. In particular, *in vivo* models have also previously been able to successfully predict the *in vivo* osteogenic effects of compounds such as BMP and insulin like growth factors (IGF). Specifically, it has been demonstrated that the osteogenic effects of BBP in an animal model using rat femur, ectopic bone formation model. Therefore it is anticipated that, based on these similar findings, BBP will have osteogenic effects *in vivo* in humans. Demonstration of osteogenic effects of a compound in these *in vivo* models are necessary prior to trials that would demonstrate their effects *in vivo* humans.

[0063] Agents which may be useful in this invention to effect osteogenesis include, but are not limited BBP alone or in combination with other osteogenic agents, such as BMP. The invention may further include any portion of the BBP peptide which is found to be active in effecting calcification or osteogenesis. The invention may further include the activation of a molecule at which the BBP is active in effecting osteogenesis. The invention may also include other peptide analogs designed to mimic the active portions of the above peptide, which would act via a similar molecular mechanism to the BBP, in effecting osteogenesis.

[0064] Therapeutically effective dose. A therapeutically effective dose of a agent useful in this invention is one which has a positive clinical effect on a patient or desired effect in cells as measured by the ability of the agent to enhance calcification or osteogenesis, as described above. The therapeutically effective dose of each agent can be modulated to achieve the desired clinical effect, while minimizing negative side effects. The dosage of the agent may be selected for an individual patient depending upon the route of administration, severity of the disease, age and weight of the patient, other medications the patient is taking and other factors normally considered by an

attending physician, when determining an individual regimen and dose level appropriate for a particular patient.

[0065] Dosage Form. The therapeutically effective dose of an agent included in the dosage form may be selected by considering the type of agent selected and the route of administration. The dosage form may include a agent in combination with other inert ingredients, including adjutants and pharmaceutically acceptable carriers for the facilitation of dosage to the patient, as is known to those skilled in the pharmaceutical arts.

[0066] Therapeutic formulations of the novel proteins may be prepared for storage by mixing the polypeptides having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; anti-oxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, blutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or poly(ethylene glycol) (PEG).

[0067] In one embodiment, the dosage form may be an oral preparation (e.g., liquid, capsule, caplet or the like) which when consumed results in the elevated levels of the agent in the body. The oral preparation may comprise carriers including dilutents, binders, time release agents, lubricants and disinigrants.

[0068] The dosage form may be provided in a topical preparation (e.g., lotion, crème, ointment, transdermal patch, or the like) for dermal application. The dosage form may also be provided in preparations for subcutaneous (such as in a slow-release capsule), intravenous, intraparitoneal, intramuscular or respiratory application, for example.

[0069] Any one or a combination of agents may be included in a dosage form. Alternatively, a combination of agents may be administered to a patient in separate dosage forms. A combination of agents may be administered concurrent in time such that the patient is exposed to at least two agents for treatment.

[0070] Additional Agents. The invention may include treatment with an additional agent which acts independently or synergistically with BBP to enhance calcification osteogenesis. For example, BBP may be combined with BMP, bisphosphonates, hormone therapy treatments, such as estrogen receptor modulators, calcitonin, and vitamin D/calcium supplementation, PTH (such as Forteo or teriparatide, Eli Lilly, sodium fluoride and growth factors that have a positive effect on bone, such as insulin-like growth factors I and II and transforming growth factor beta. Those skilled in the art would be able to determine the accepted dosages for each of the therapies using standard therapeutic dosage parameters, or reduced dosages where the effects of BBP are synergistic with the secondary agent.

[0071] FIG. 12 depicts a flowchart of one method according to this invention. In this embodiment of the method, mammalian cells, such as mesenchymal stem cells can be harvested, form the patient or a cell donor (100). The cells may be injected where bone formation or repair is desired, or first treated with at least one agent to induce osteogenesis or specifically osteoblastic differentiation (102). The cells may then be re-administered to the patient, either systemically or at a selected site at which osteogenesis is desired (104). Additionally, the patent may by treated locally or systemically with at least one additional agent which effects osteogenesis, such as BBP alone or in combination with BMP or demineralized bone matrix (106).

[0072] For example, FIGS. 13A & B depict two embodiments of the present invention. In FIG. 13A, the invention may include implants or grafts (200) for use in the human body comprising, a substrate having a surface (201), wherein at least the surface of the implant includes BBP (203) in an amount sufficient to induce chondrogenesis, calcification or osteogenesis in the surrounding bone tissue, or implant includes osteogenic mammalian cells expressing BBP, and or BMP. The implant may also include, but are not limited to pins, screws, plates or prosthetic joints which may be

placed in the proximity of or in contact with a bone (202) that are used to immobilize a fracture, enhance bone formation, or stabilize a prosthetic implant by stimulating formation or repair of a site of bone removal, fracture or other bone injury (204).

[0073] As shown in FIG. 13B, the invention may also include the *in vitro* (such as on cultures of collagen or chondrocytes) *or in vivo* application of at least BBP containing composition or BBP expressing cells (206) in the proximity of or in contact with a bone (202), an implant (200) at a site of bone removal, fracture or other bone injury (204) where calcification or osteogenesis is desired. The BBP composition may be applied in combination with other agents such as BMP-2, deminieralized bone matrix, collegen cultures

[0074] For example: stem cells for treating bone related disorders in humans has also been examined. Infusion of osteoblastic progenitor stem cells from a healthy individual into a diseased individual has been shown to improve bone density in these patients (OI). However, additional substances would be valuable.

[0075] Example 1

[0076] A. EXTRACTION AND SEPARATION OF NONCOLLAGENOUS BONE PROTEINS.

[0077] Methods: NCPs were extracted from defatted, demineralized human cortical bone powder with 4 M GuHCl, 0.5 M $CaC1_2$, 2 mM N-ethylmalemide, 0.1 mM benzamidine HCl, and 2 mM NaN_3 for 18 hr at 6° C. Residual collagen and citrate-soluble NCPs were extracted by dialysis against 250 mM citrate, pH 3.1 for 24 hours at 6° C. The residue was pelleted by centrifugation (10,000 x g at 6° C for 30 min), defatted with 1:1 (v/v) chloroform: methanol for 24 hr at 23° C, collected by filtration and dried at 22° C. The material was resuspended in 4 M GuHCl, dialyzed against 4 M GuHCl, 0.2% (v/v) Triton X-100, 100 mM Tris-HCl, pH 7.2 for 24 hr at 6° C, then dialyzed against water, and centrifuged at 10,000 x g for 30 min at 6° C. The pellet was lyophilized and subsequently separated by hydroxyapatite chromatorgraphy.

[0078] Chromatography was conducted using a BioLogic chromatography workstation with a CHT-10 ceramic hydroxyapatite column (BioRad, Hercules, CA).

Bovine BMP/NCP was solublized in 6 M urea, 10 mM sodium phosphate, pH 7.4. The sample was loaded onto the hydroxyapatite column and the unbound fraction was collected. Bound proteins were eluted with increasing concentration of sodium phosphate to 300 mM over a linear gradient of five column volumes. Five ml fractions were collected during the course of the run. The fraction which separated at 180 mM phosphate was separated further by SDS-PAGE electrophoresis. A band corresponding to a M_r of 18.5 was excised and submitted for sequence analysis by matrix assisted laser-desorption ionization/time of flight mass spectroscopy (MALDI/TOF MS).

[0079] Results: The fraction of bBMP/NCP which eluted from hydroxyapatite at 180 mM phosphate was separated by SDS-PAGE electrophoresis and the material with a M_r of 18.5 kD was submitted for MALDI/TOF MS analysis. The major protein component of this material was determined to be a fragment of SPP-24 on the basis of six peptides with sequences identical to regions of that protein. [3] The sequences of these peptides are shown in Table 1.

[0080] Table 1. Identification of the 18.5 kD protein by MALDI/TOF mass spectroscopy and peptide fingerprinting.

Expected Mass [a]	Observed Mass [a]	Peptide Sequence
1526.574	1526.53	ESEADPATCDFQR *
1411.600	1411.71	VNSQSLSPYLFR
1291.406	1291.41	SRGEPLYEPSR
1249.409	1249.48	NSYLLGLTPDR
1158.363	1158.27	GYHVPVAVCR *

* modified cystein; a = peptide masses are expressed as $[M + H^+]$

[0081] Analysis of this sequence with the SWISS-PROT data base revealed the cystatin-like domain which had been previously described, but no other sequence similarities of relevance to bone metabolism. [3] However, it is known from other work that other cystatin-like proteins do interact with proteins that are important in bone metabolism. Specially, others have shown that a member of the cystatin family has TGF-β and BMP-2 binding properties based on similarities to the TGF-β receptor.[1, 2]

However fetuin appears to antagonize BMP activity. (3) Therefore, we undertook a manual comparison of the cystatin-like region of SPP-24 and the cystatin-like domain of fetuin. Two regions of interest were identified in the cystatin-like region of SPP-24. One region has some sequence similarity to BMP-2 whereas the other region has sequence similarity to the TGF-β receptor II homology domain of fetuin. That part of the sequence of SPP-24 which contains these two regions is shown in Figure 1. Comparisons of the two regions of interest to human BMP-2 and human TGF-β receptor II are shown in Figures 2 and 3. Alignment of the SPP-24, fetuin, human BMP-2, and human TGF-β receptor II sequences was accomplished using the T-Coffee program accessed over the internet (http://www.ch.embnet.org/software/Tcoffee.html). [5] Synthetic peptides corresponding to these two regions were obtained and subjected to chemical and *in vivo* analysis as described below.

[0082] B. *IN VIVO* ASSAY TO DETERMINE THE ACTIVITY OF SYNTHETIC PEPTIDES.

[0083] Methods: The osteogenic activity of material was tested using an *in vivo* assay approved by the Sepulveda Animal Subjects Committee and the VA Greater Los Angeles Research and Development Committee. Male Swiss-Weber mice aged 8 to 10 weeks were used (Taconic Farms, Germantown, NY). Prior to the assay, the material to be tested was solublized and lyophilized into 2 mg of atelocollagen. The dried material was placed in a #5 gelatin capsule and sterilized by exposure to chloroform vapor. To conduct the assay, mice were anesthetized using 1% isoflurane delivered in oxygen at 2 l/min through a small animal anesthesia machine (VetEquip, Pleasanton, CA). Animals were affixed to a surgery board and the fur over the hindquarters shaved. The skin was cleaned with 70% ethanol and a midline incision made over the spine adjacent to the hindquarters. Blunt dissection with scissors was used to expose the quadriceps muscle on one side. A small pouch was made in the muscle using the point of scissors and the #5 capsule containing the test material was inserted into the pouch. The skin was then closed with three 11 mm Michel surgical clips and the animal returned to its cage for monitoring. After 21 days the animals were

killed and the hindquarter removed. Radiological examination of the specimens was accomplished using a small parts X-Ray cabinet (Faxitron, Wheeling, IL). For quantitation of bone formation, bone area and the bone mineral content (BMC) of an area of interest encompassing the site of ectopic bone formation was determined using a PIXImus2 small animal densitometer (GE Lunar, Madison, WI). Specimens were then placed in buffered formalin and submitted for routine processing for histological examination.

[0084] Various amounts of rhBMP-2 and the synthetic peptide (see below) were mixed together and prepared for implantation. All possible combinations of the following amounts were used in pilot studies, rhBMP-2: 0 μg, 0.05 μg, 0.5 μg, 5 μg, and 50 μg; peptide: 0 μg, 50 μg, and μg 500 mg. Samples of 5 μg of rhBMP-2 were used in more extensive subsequent studies because that amount consistently produced an amount of ectopic bone that was neither too large nor too small for reliable analysis.

[0085] **Results:** An aliquot of 0.5 mg of the peptide with sequence homology to BMP-2 was lyophilized into 2 mg of atelocollagen carrier and tested in the standard *in vivo* assay for osteogenic activity. Four animals were tested and no ectopic bone formation was detected.

[0086] The peptide with sequence similarity to the TGF-β receptor II was tested alone and in combination with BMP-2. When implanted alone with carrier, the peptide induced calcification which was apparent radiologically as seen in Figure 4. On histological examination, dystrophic calcification, primarily in association with intra-muscular adipose tissue, was seen in animals implanted with the peptide but not in carrier alone control animals (Figure 5).

[0087] When 500 μg of the peptide with sequence similarity to the TGF-β receptor II was implanted with 5 μg of rhBMP-2 the amount of ectopic bone formed, as measured by densitometry, was consistently greater than the amount of bone formed in animals into which identical amounts of the rhBMP-2 alone were implanted. This is seen radiologically in Figure 6 and the quantitative densitometric data is shown in Table 2. Furthermore, implants that contained both the peptide and rhBMP-2 produced

detectable cartilage and bone earlier than implants of BMP-2 alone. This is shown radiologically in FIG. 7 and histologically in FIG. 8.

[0088] Table 2. Densitometric quantitation of ectopic bone formation with various amounts of BBP implanted with 5 μg of rhBMP-2. Mean, SE (n).

Peptide Amount (μg)	0	50	500
Bone Area (cm^2)	0.089 ± 0.0336 (12)*	0.159 ± 0.0606 (8)	0.226 ± 0.0270 (12)*
Bone Mineral Content (g)	0.00189 ± 0.00084 (12)**	0.00388 ± 0.0017 (8)	0.00528 ± 0.00068 (12)**

[0089] * p = 0.0044; ** p = 0.0049

[0090] C. SURFACE PLASMON RESONANCE TO DETERMINE THE INTERACTION OF BMP-2 AND THE SYNTHETIC PEPTIDE.

[0091] **Methods:** The binding interaction between rhBMP-2 and the synthetic peptide was characterized using surface plasmon resonance employing a Biacom X instrument (Biacore, Piscataway, NJ). Buffers and chips for the procedure were obtained from Biacore. RhBMP-2 was dialyzed into 10 mM sodium acetate, pH 5.5 at a concentration of 1 mg/ml. This material was then attached to a CM-5 sensor chip using reagents and procedures supplied by the manufacturer. Running buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20. The peptide was dissolved in running buffer at concentrations ranging from 1×10^{-5} to 1×10^{-4} M. Flow rates from 5 to 50 μl/min and injection volumes of 20 to 100 μl were employed. The regeneration solution was 10 μM glycine-HCl, pH 2.0.

[0092] **Results:** Results of the surface plasmon resonance studies to determine the interaction between rhBMP-2 and the synthetic peptide with sequence similarity to the TGF-β receptor II are shown in Figures 9. The estimated dissociation constant (K_D) for the interaction was 3×10^{-5} M. When the peptide was decyclized by prior reduction with β-mercaptoethanol, no significant binding occurred (data not shown).

[0093] D. PLASMID CONSTRUCTION FOR THE GENERATION OF TRANSGENIC ANIMALS

[0094] Methods: The sequence of the major constituent was determined to be identical to that of a fragment of Secreted Phosphorotein 24 (SPP-24) with a M_r of 18.5 kD. The entire sequence for this protein was located in Genbank and was used to produce an expression plasmid for the generation of transgenic mice. Bovine liver cDNA was prepared from bovine polyA$^+$ RNA using a MasterAmp RT-PCR kit (Epicentre, Madison, WI). The cDNA was amplified using the following primers: [gcgttttctagagccaccatggcgatgaagatgttggtg] and [GCGTTTACCGGTCTCAAAGCCAGGGTTTACTC]. This material was then digested with XbaI-AgeI and cloned into plasmid pcDNA3.1V5HisA (Invitrogen, Carlsbad, CA) . The sequence of the 24 kb was confirmed using vector primers. The 24 kb insert was then amplified using the same primers as shown above and digested with BglII-BpiI. Plasmid pOC-IGF-I (kindly supplied by the laboratory group of Thomas Clemens, Ph.D.) [12] was digested with BamHI-EcoRI and the 4.9 kb EcoRI-BamHI and the 2.0 kb BamHI-BamHI fragments were gel purified. The smaller fragment was dephosphorylated. The following adapter oligos were annealed: [tagcaggcatgctgggga] and [aatttccccagcatgcct]. Ligation was then performed combining the following components: the 4.9 kb EcoRI-BamHI fragment of pOC-IGF-I; the 2.0 kb deposphorylated BamHI-BamHI fragment of pOC-IGF-I; the 0.85 kb BglII-BpiI amplification product; and the annealed BpiI-EcoRI compatible adapter oligos. The products were screened for the proper orientation of the 2.0 kb fragment by digestion with EcoRI-XhoI which produced a 780 bp fragment. Sequencing was then conducted to confirm the sequence of the insert as well as the OC (osteocalcin) promoter and rabbit β-globin portions of pOC-IGF-I plasmid. Sequence information was compared to Genbank and an OC promoter-β-globin clone (AB004306) and a human chromosome 1 clone (AL135927, clone RP11-54H19) were used to make corrections to the OC promoter and β-globin portions of the new plasmid, pOC-24kd. This plasmid was digested with SapI-HindIII and the gel purified fragment was sent to the Transgenic

Mouse Facility at the University of California, Irvine for the generation of transgenic mice.

[0095] PRODUCTION AND DETECTION OF TRANSGENIC MICE. Transgenic mice were generated in FVBn mice at the UCI Transgenic Mouse Facility using their protocols (http://darwin.bio.uci.edu/~tjf/index.html). Five founders were obtained. These animals were crossed with wild type FVBn mice and animals carrying the transgene were identified by polymerase chain reaction (PCR) of DNA extracted from tissue obtained from the tail. DNA was extracted using a DNeasy kit (Qiagen, Valencia, CA). PCR was conducted using a HotStar DNA polymerase kit (Qiagen, Valencia, CA). The PCR primers used were identical to those employed by other investigators (M. Zhang, personal communication): (osteocalcin promoter region) 5'CAAATAGCCCTGGCAGATTC3' and (rabbit β globin region) 5'TGATACAAGGGACATCTTCC3'. The expected band of 230 bp was visualized by agarose gel electrophoresis. The PCR protocol involved 30 cycles of melting (94 C, 0.5 min.), annealing (53° C, 0.5 min.) and extention (72° C, 0.5 min.) and was conducted in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). Heterzygous animals of both genders and from all lines were used for the studies described here.

[0096] HISTOMORPHOMETRY. To examine bone turnover in cortical bone, dynamic histomorphometric analyses were performed on the femoral metaphysis of heterozygous transgenic mice and non-transgenic litermates. Bone turnover in trabecular bone was examined by performing histomorphometrical analysies on the cancellous bone in an area between the lowest point of the growth plate and 1.5 mm distal to the growth plate. Labeling was accomplished with i.p. injections of 20 mg/kg of tetracycline HCl on days 1 and 2 and on days 9 and 10. Animals were killed on day 12 and one femur from each animal was excised. The soft tissue surrounding the bone was removed and the specimens were placed in 70% ethanol and stored at 4° C. The femurs were dehydrated in graded ethanol, defatted in xylene, and embedded without demineralization in methyl methacrylate. Five micrometer thick coronal sections were cut parallel to the long axis of the femur, using a Leica RM 2155 microtome (Nussloch, Germany) and stained with Goldner's trichrome. Additional twelve-micrometer-thick

unstained sections were prepared for observations under UV light. The following histomorphometric parameters were recorded in these standard sampling sites using the guidelines of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee: (1) trabecular bone volume (BV/TV, %); (2) mineral apposition rate (MAR, μm/day); (3) bone formation rate (BFR, %/yr); (4) trabecular thickness (Tb.Th, μm); (5) trabecular separation (Tb.Sp, μm); and (6) trabecular number (Tb.N, per mm). [6] Histomorphometric measurements of bone structure and microarchitectural parameters in cancellous bone were performed bone using a semiautomatic color image analyzer (ImagePro Plus, Version 4.5.1, Media Cybernetics, Silver Springs, MD) attached to a personal computer.

[0097] BONE DENSITOMETRY. Bone density measurements were performed using a PIXImus2 densitometer designed specifically for use with small animals (GE Lunar Corp., Madison, WI). Killed animals were placed in the specimen tray and, after appropriate calibration, duplicate cycles of four scans were obtained. Data from three regions of interest were obtained. "Total body" included all highly dense (osseous) tissues other than the skull and tail which are routinely excluded. "Vertebral" was defined by a rectangle that extended from end plate to end plate for the five center-most thoracolumbar vertebrae and which included the lateral margins of all of the vertebrae involved. "Femoral" was defined as a rectangle forty pixils in length centered in the mid-shift region of the femur and which included both cortices of the bone. Densitometry was also conducted on areas of ectopic bone formation which had been dissected away from the femur of test animals.

[0098] SERUM CALCITONIN AND OSTEOCALCIN ASSAYS. Serum concentrations of calcitonin and osteocalcin were determined as described previously.[4] Blood samples were collected by heart puncture between 08:00h and 12:00h on the day that the mice were killed. Serum was obtained by centrifuging heparinized, unhemolyzed blood in a microfuge for 10 minutes at 4° C. The serum was stored at -80° C until the assays were performed. Serum concentrations of peptides were determined using commercial radioimmunoassay (RIA) kits for calcitonin (Incstar, Stillwater, MN) and osteocalcin (Biomedical Technologies, Stoughton, MA). All assays

were conducted in triplicate in multiple dilutions of serum and with control serum using high and low levels of the peptides. Assay results were calculated using AssayZap software (Biosoft, Ferguson, MO).

[0099] NUMERICAL ANALYSIS. Means of treatment groups were compared with Student's t-test using InStat software (GraphPad Software, San Diego, CA). All numerical data is presented as mean ± SE, standard error of the mean, (n), sample size.

[00100] Results: Five independent strains of transgenic mice were generated in which the entire sequence of SPP-24 was expressed under the control of the osteocalcin promoter. Founder animals were crossed to normal animals and heterozygous animals identified by analysis of tail DNA. The bones of heterozygous animals were analyzed at 3 and 8 months of age by densitometry and histomorphometry. The results of the densitometric analysis are shown in Tables 3A and B. Female transgenic animals consistently showed a reduced femoral bone mineral density (BMD) as compared to non-transgenic littermates at both 3 and 8 months whereas male animals did not. Female transgenic animals also had a reduced vertebral BMD at 3 months but not at 8 months. The vertebral BMD of transgenic male animals was not different from that of non-transgenic littermates.

[00101] Table 3A. Bone mineral density (g/cm^2) of wild type and transgenic animals at three months of age. Mean ± S.E. (n).

	Femur BMD	Vertebral BMD
Gender/Genotype		
Female/Wild Type	.0730 ± .0022 (5)	.07286 ± .0041 (5)
Female/Transgenic	.0654 ± .0017 (8)	.0585 ± .0006 (8)
p value WT v. Transgenic	.018	.0011
Male/Wild Type	.0676 ± .0022 (5)	.0563 ± .0009 (5)
Male/Transgenic	.0715 ± .0043 (6)	.0616 ± .0037 (6)
p value WT v. Transgenic	.47	.24

[00102] Table 3B. Bone mineral density (g/cm^2) of wild type and transgenic animals at eight months of age. Mean ± S.E. (n).

	Femur BMD	Vertebral BMD
Gender/Genotype		
Female/Wild Type	.0676 ± .0009 (15)	.0605 ± .0016 (17)
Female/Transgenic	.0637 ± .0015 (7)	.0591 ± .0018 (10)
p value WT v. Transgenic	.0258	.5722
Male/Wild Type	.0600 ± .0018 (12)	.0520 ± .0014 (14)
Male/Transgenic	.0609 ± .0016 (8)	.0546 ± .0024 (9)
p value WT v. Transgenic	.6094	.3215

[00103] The microscopic anatomy of femurs from transgenic mice and non-transgenic littermates is shown in figure 10A and B and 11A and B. The quantitative histomorphometric analyses are shown in Table 4. Reduced trabecular mass accompanied by decreased connectivity and increased separation is apparent in the transgenic animals in comparison to their non-transgenic littermates. As in the case with the densitometric measurements, the differences in bone structure were seen primarily in female animals.

[00104] Table 4. Histomorphometric analysis of femurs from wild type and transgenic animals. Mean ± S.E. (n).

Parameter	Gender	Wild Type	Transgenic	p value WT v. Trans
Bone Volume (%)	female	15.68 ± 2.19 (5)	7.82 ± 1.18 (7)	.0066
	male	10.57 ± 1.92 (5)	10.21 ± 0.90 (7)	.85
Mineral Apposition Rate (μm/day)	female	1.03 ± .157 (3)	0.701 ± .166 (5)	.24
	male	2.14 ± .527 (3)	1.77 ± .092 (5)	.39
Bone Formation Rate (%/year)	female	37.47 ± 5.72 (3)	32.63 ± 3.12 (5)	.44
	male	77.99 ± 19.36 (3)	64.50 ± 3.37 (5)	.40
Trabecular Thickness (μm)	female	42.37 ± 4.68 (5)	25.15 ± 2.40 (6)	.0072
	male	38.01 ± 4.85 (5)	32.97 ± 2.11 (7)	.31
Trabecular Number (/mm)	female	.36 ± .024 (5)	.34 ± .058 (6)	.74
	male	.31 ± .089(5)	.31 ± .029 (7)	.98
Trabecular Separation (μm)	female	657.61 ± 131 (5)	160.01 ± 39.1 (5)	.0361
	male	341.6 ± 54.1 (5)	307.0 ± 39.2 (7)	.61

[00105] Serum concentrations of osteocalcin and calcitonin are shown in Table 5. Serum osteocalcin was lower in female transgenic animals. There were no other significant differences.

[00106] Table 5. Serum concentrations of calcitonin and osteoclacin in wild type and transgenic animals. Mean ± S.E. (n).

Parameter	Gender	Wild Type	Transgenic	p value WT v. Trans
Calcitonin (pg/ml)	female	183.8 ± 17.8 (5)	211.8 ± 25.8 (6)	.41
	male	209.8 ± 30.0 (5)	184.7 ± 21.2 (7)	.50
Osteocalcin (ng/ml)	female	36.4 ± 2.95 (5)	27.9 ± 1.79 (7)	.0256
	male	24.1 ± 3.65 (4)	25.5 ± 3.20 (7)	.80

[00107] While the specification describes particular embodiments of the present invention, those of ordinary skill can devise variations of the present invention without departing from the inventive concept.

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BONE MORPHOGENIC PROTEIN (BMP) BINDING PROTEIN

ABSTRACT OF THE DISCLOSURE

A peptide designated "BMP Binding Protein" (BBP) is a secreted peptide. This peptide has calcifying activity alone, and acts synergistically with BMP to increase the rate and amount of osteogenesis. This peptide and the method of using the peptide can be useful in therapeutic, diagnostic and clinical applications requiring calcification and osteogenesis.

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SEQ ID No 1a: Cys-Arg-Ser-Thr-Val-Arg-Met-Ser-Ala-Glu-Gln-Val-Gln-Asn-Val-Trp-Val-Arg-Cys

SEQ ID No 1b: TGC-AGA-AGC-ACC-GTG--CGG-ATG--TCT--GCT-GAA-CAG-GTG-CAG-AAC--GTG-TGG-GTT-CGC--TGC

FIGURE 1A

FIGURE 1B

cystatin homology region

leader sequence BMP-2 homology region

(1) MAMKMLVIFVLGMNHWTCTGFPVYDYDPASLKEALSASVAKVNSQSLSPYLFRAFRSSVKRVNALDEDSLTMDLE (75)

cystatin homology region

TGF-β receptor II homology region

(76) FRIQETTCRRESEADPATCDFQRGYHVPVAVCRSTVRMSAEQVQNVWVRCHWSSSSGSSSSEEMFFGDILGSSTS (150)

(151) RNSYLLGLTPDRSRGEPLYEPSREMRRNFPLGNRRYSNPWPRARVNPGFE (200) **SEQ ID No 1c**

Figure 2

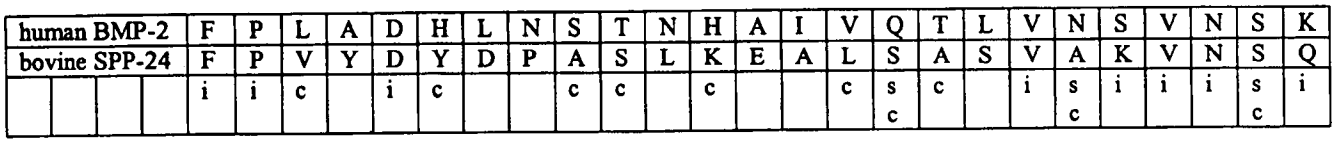
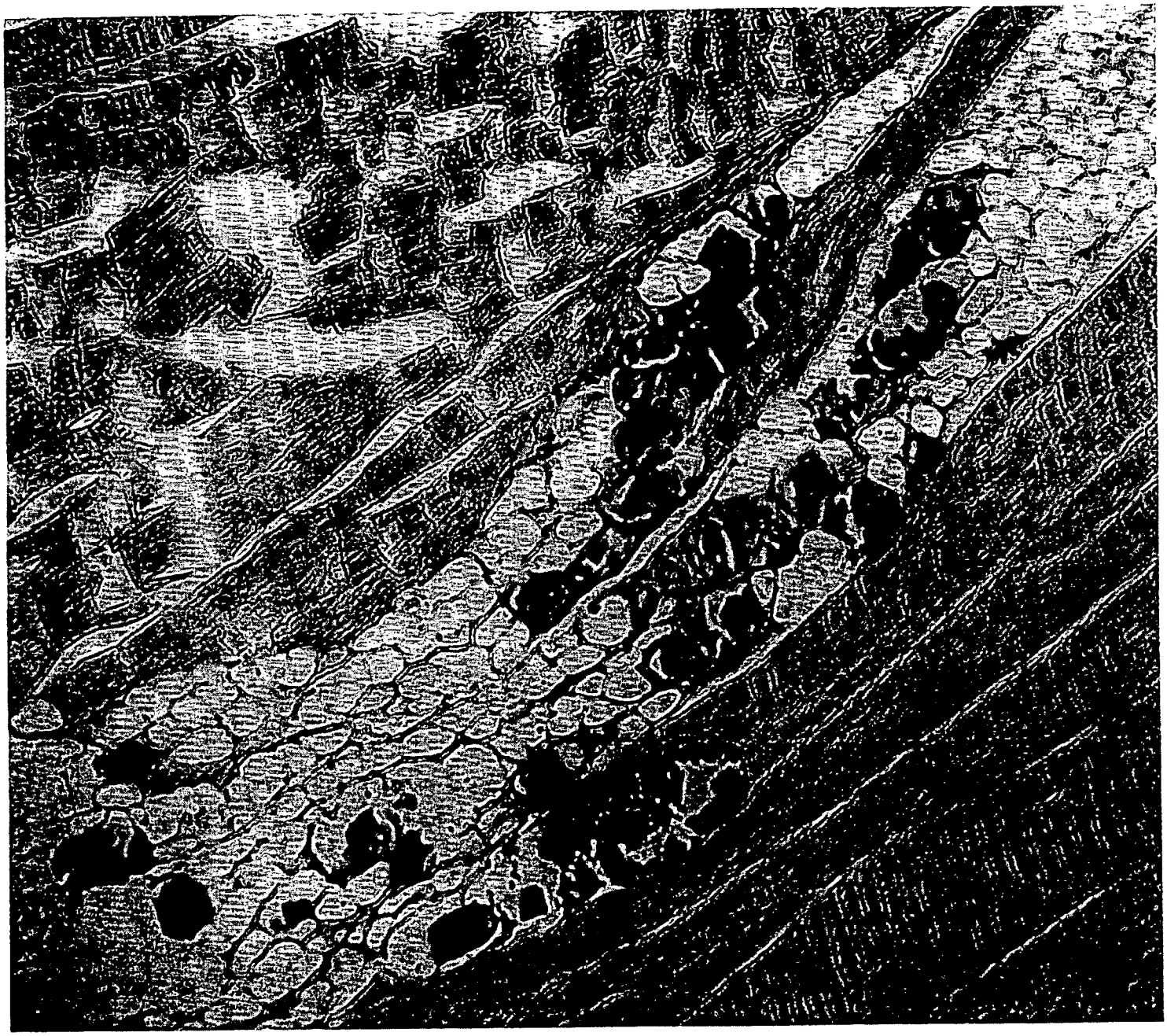

human BMP-2 F P L A D H L N S T N H A I V Q T L V N S V N S K
bovine SPP-24 F P V Y D Y D P A S L K E A L S A S V A K V N S Q
i i c i c c c c c i s c i i i s c i
c s c s c

Figure 3

bovine fetuin	114	C	D	I	H	V	L	K	Q	D	G	Q	F	S	V	L	F	T	K	C		
human TGF–β receptor II	84	C	*	V	A	V	W	R	K	N	D	E	N	I	T	L	E	T	V	C		
		i		c		i		c	c	c	s c	c			s c	i		i		i		
human TGF–β receptor II	84	C	V	A	V	W	R	K	N	D	*	*	*	E	N	I	T	L	E	T	V	C
bovine BBP		C	R	S	T	V	R	M	S	A	E	Q	V	Q	N	V	W	V	R	*	*	C
		i		c	s c		i							c	i	c		c	s c			i

Figure 4

Figure 5



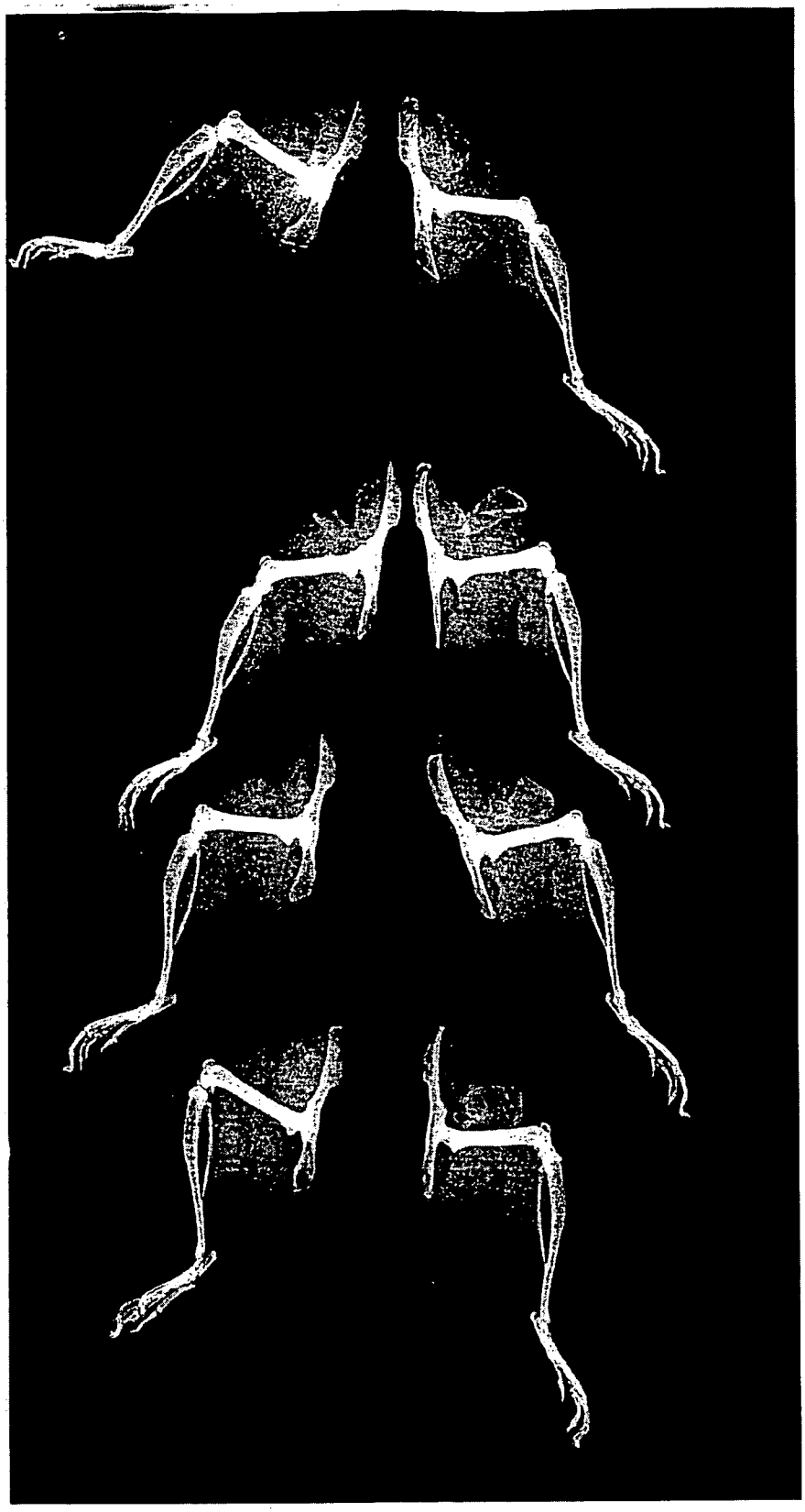


Figure 6

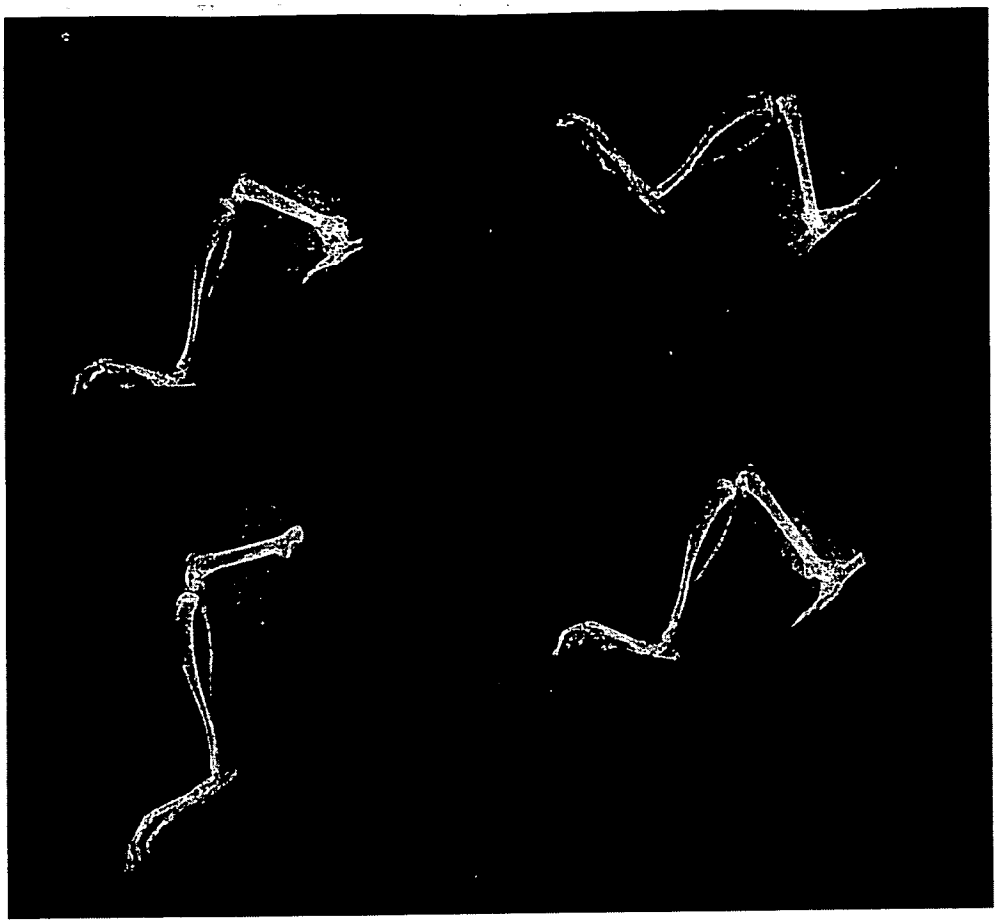


Figure 7

Figure 8A

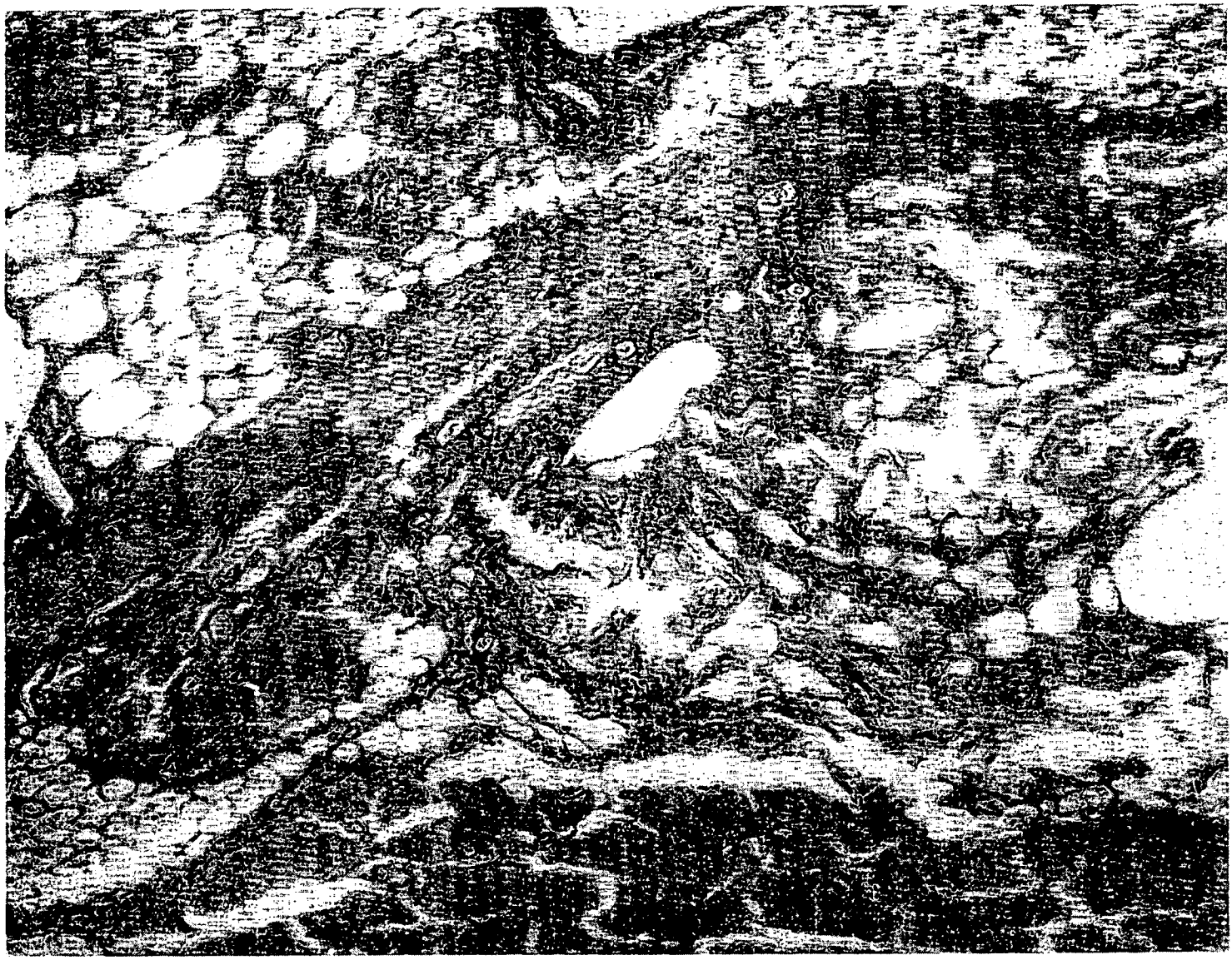
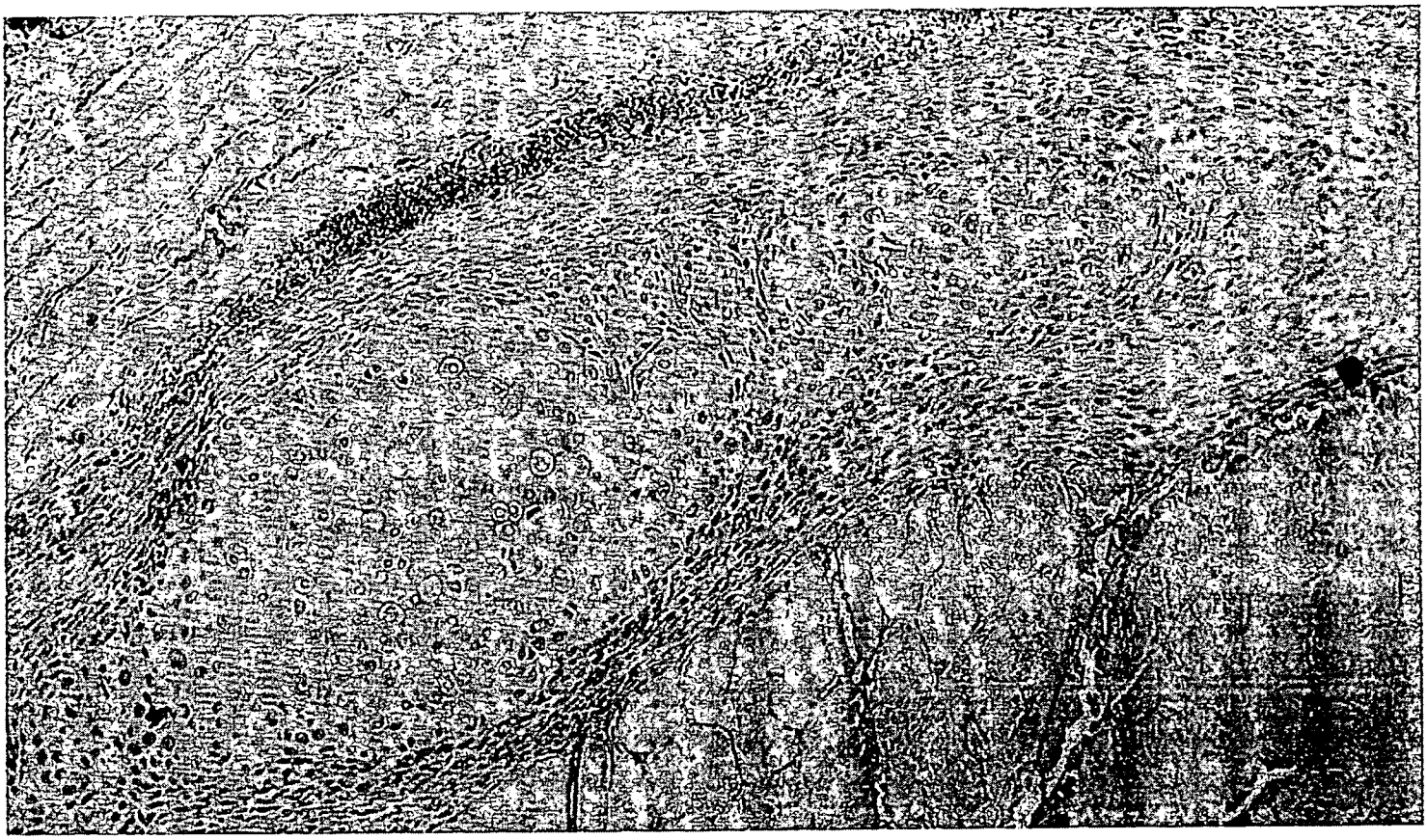


Figure 8B



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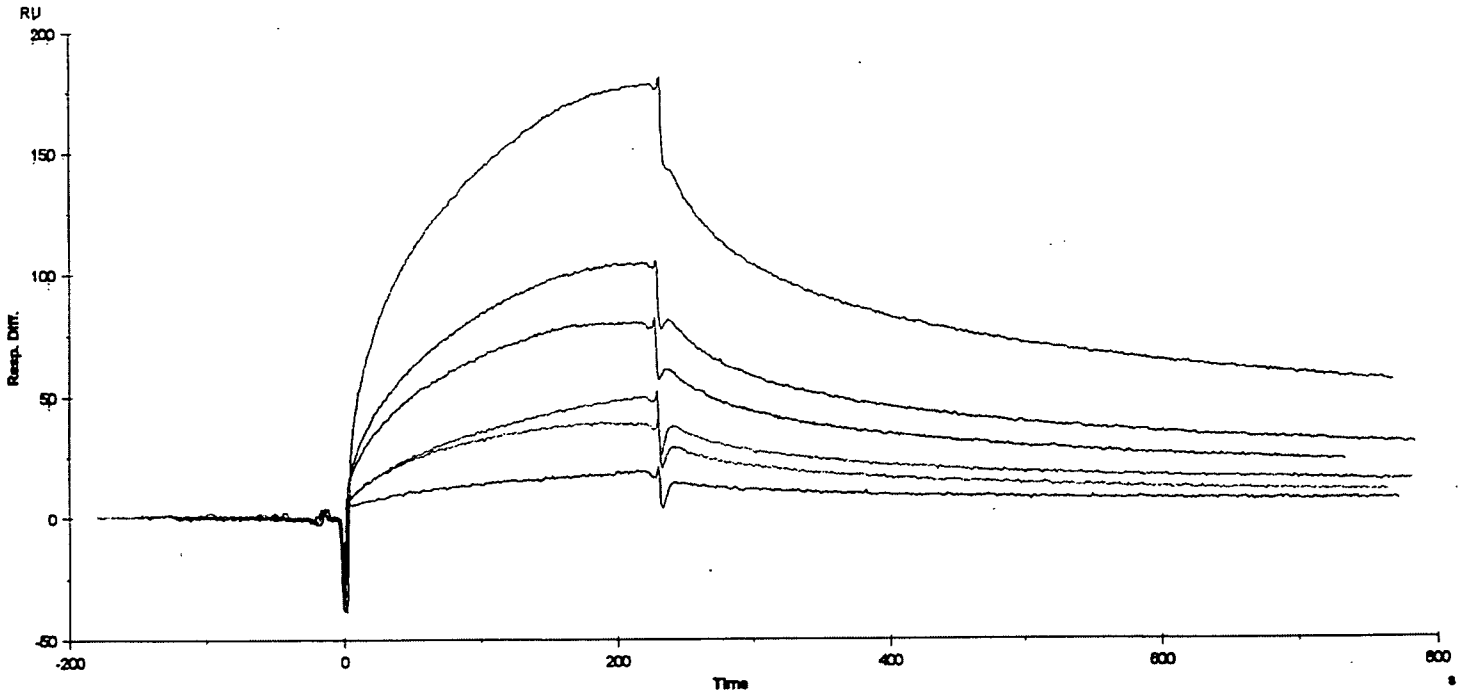


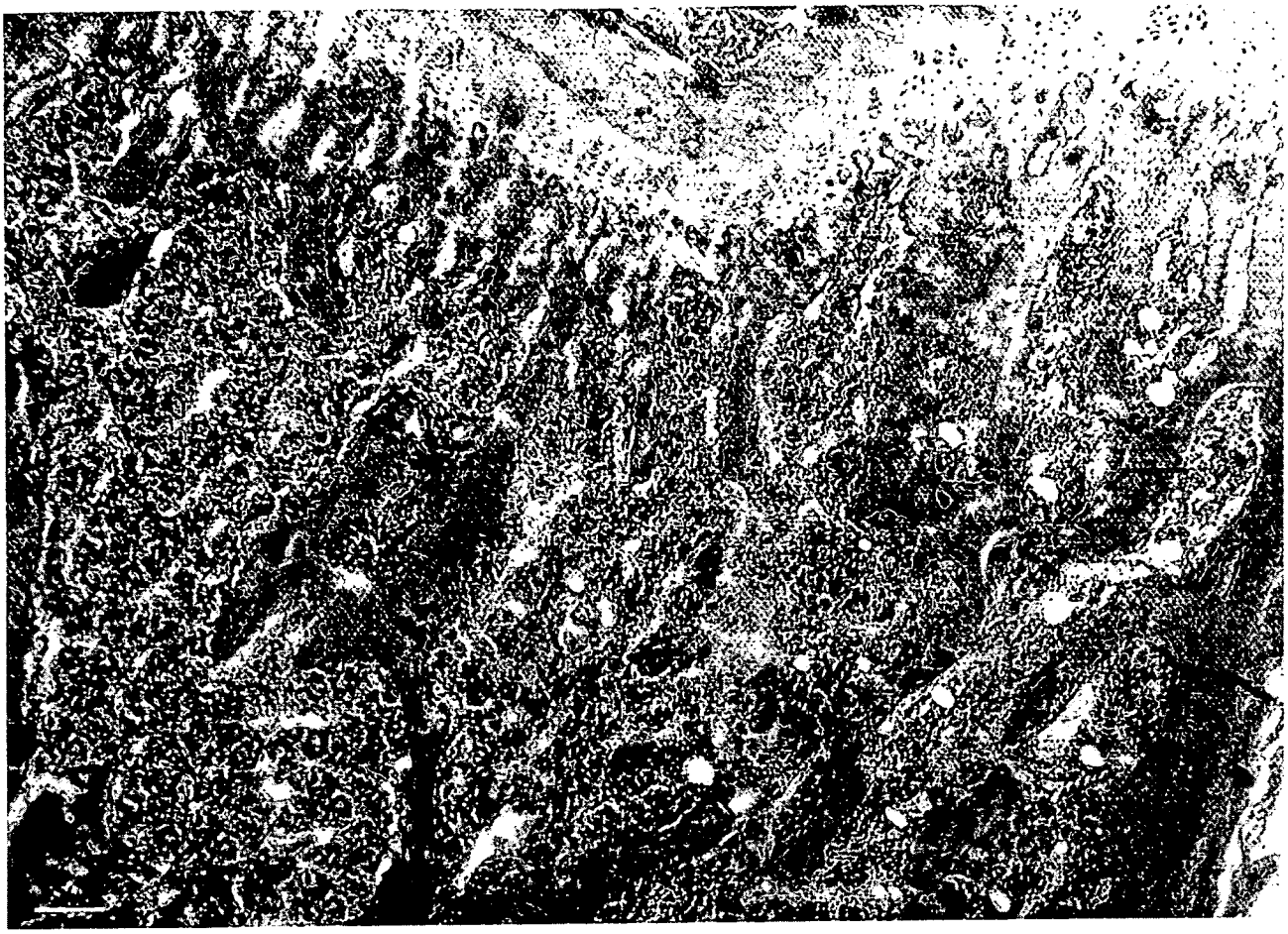


Figure 10A

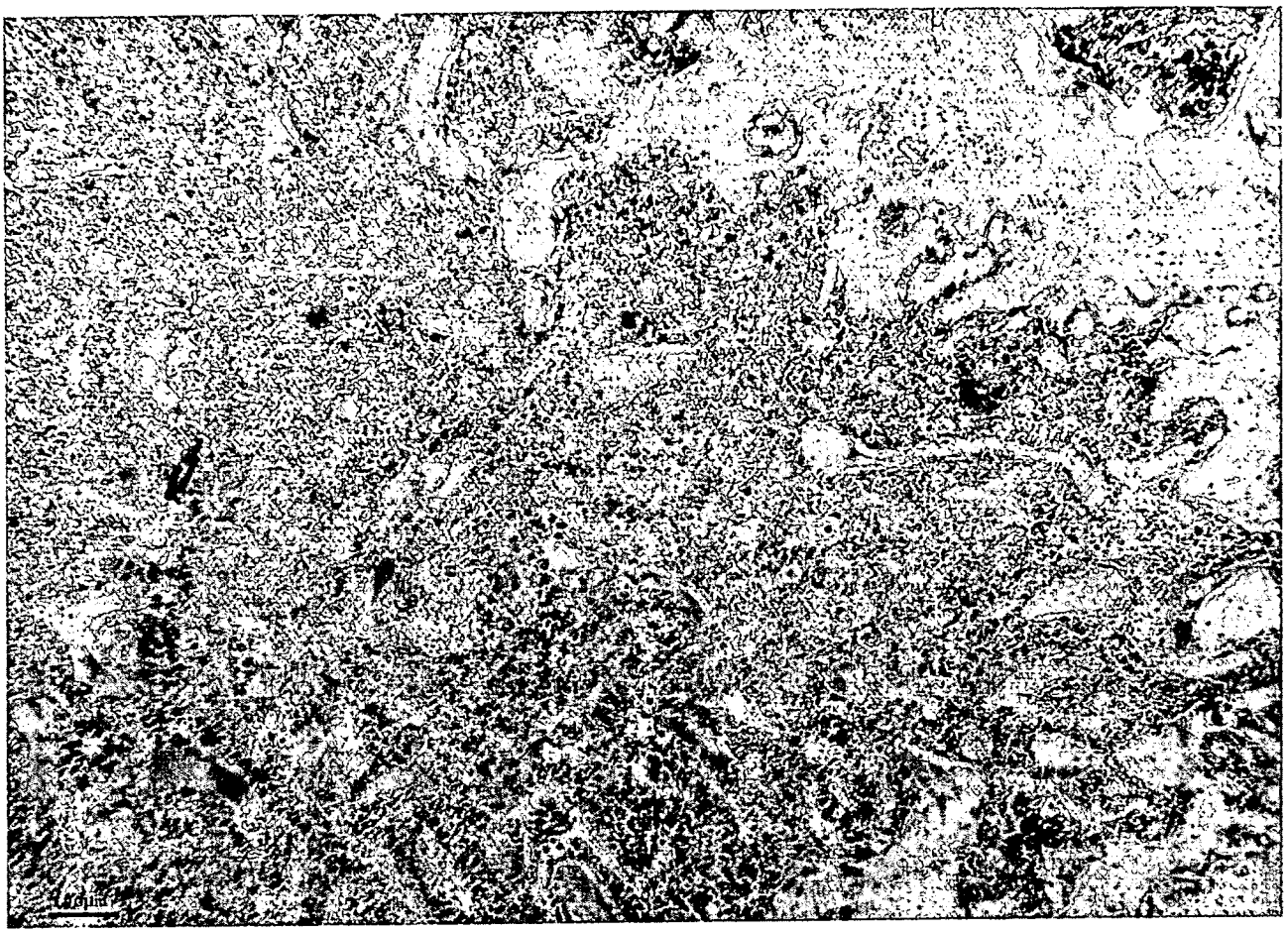


Figure 10B

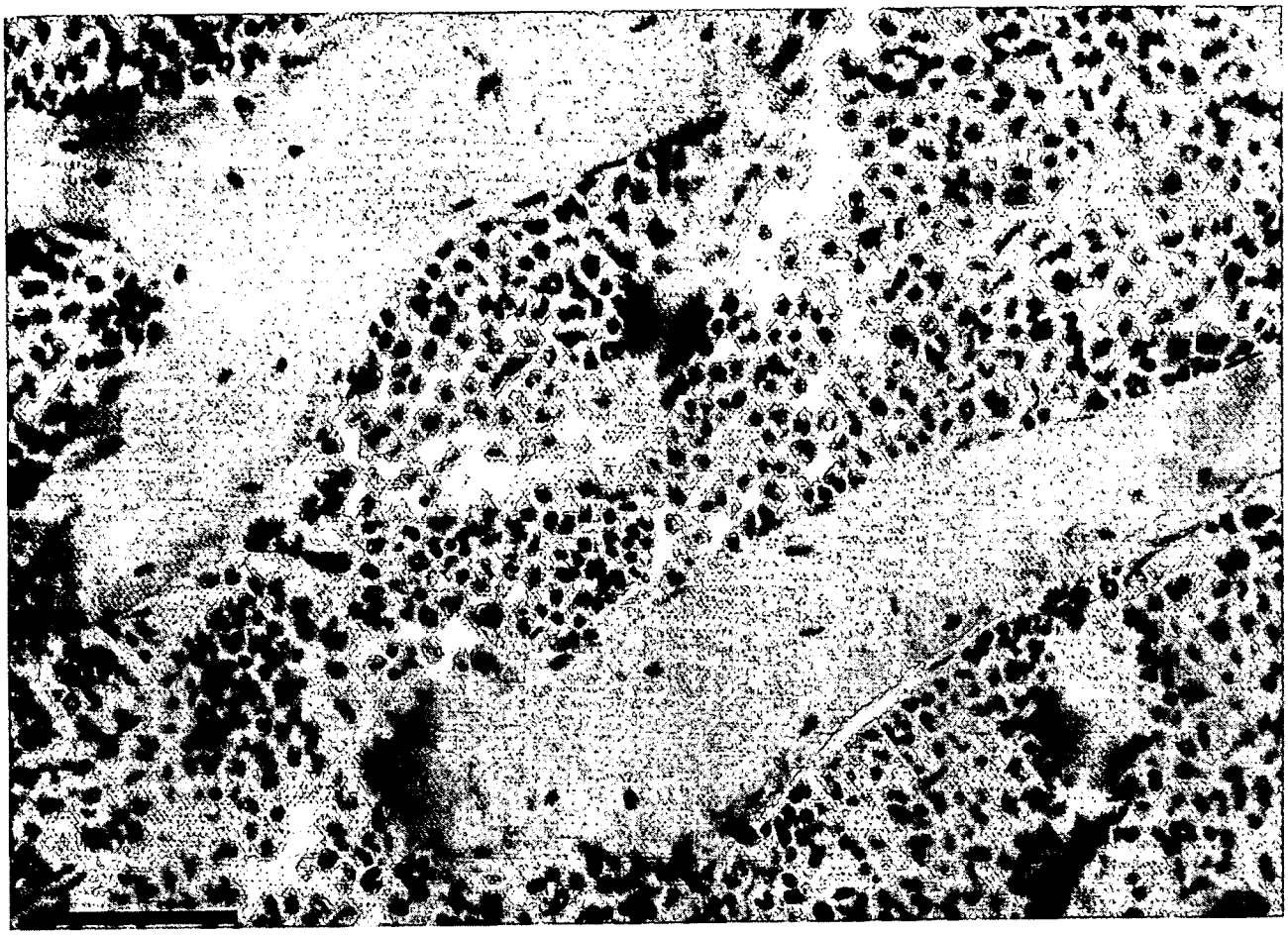


Figure 11A

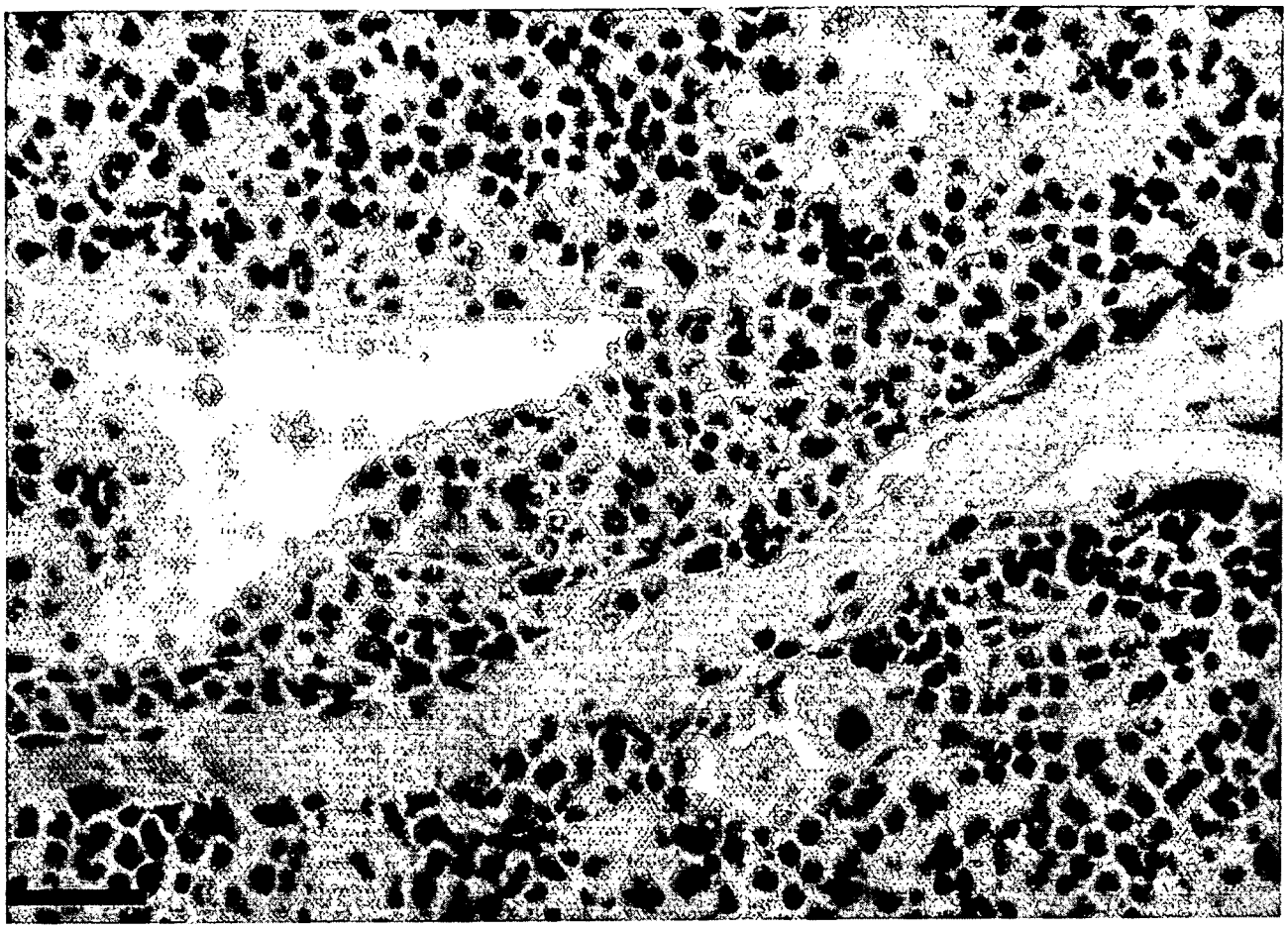


Figure 11B

FIGURE 12

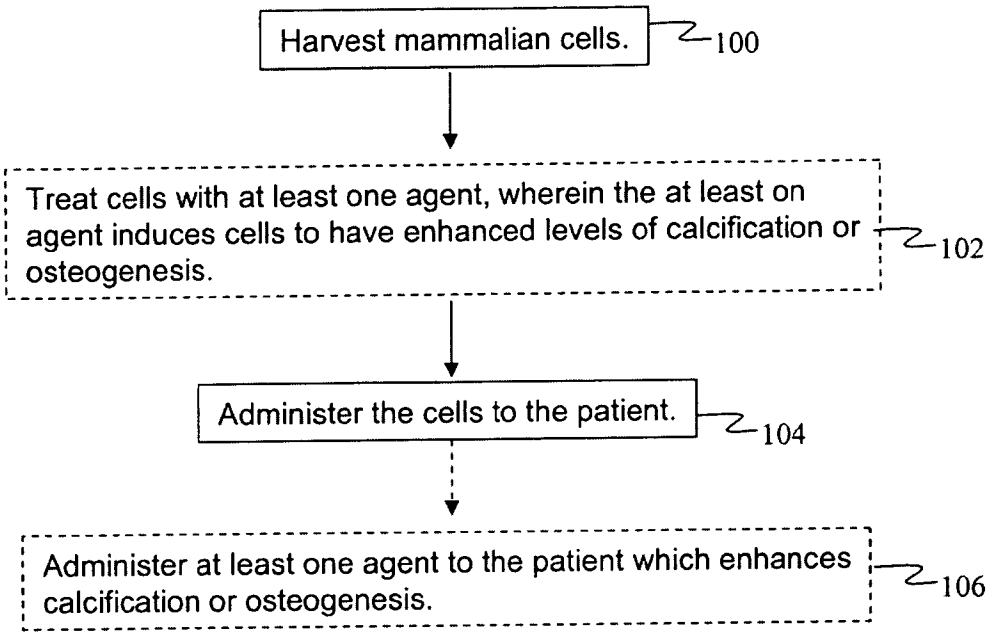
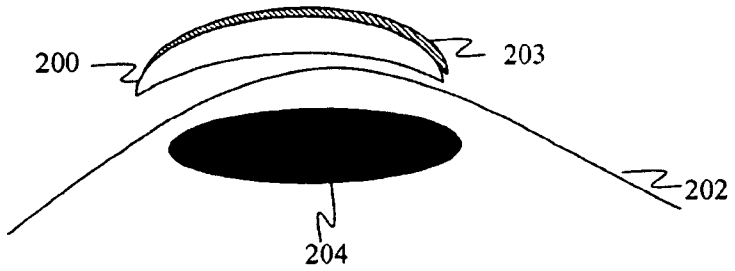

Harvest mammalian cells.
100
Treat cells with at least one agent, wherein the at least on agent induces cells to have enhanced levels of calcification or osteogenesis.
102
Administer the cells to the patient.
104
Administer at least one agent to the patient which enhances calcification or osteogenesis.
106

FIGURE 13

A



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